Proc. Nat. Acad. Sci. USA
Vol. 71, No. 4, pp. 1133–1136, April 1974

The Effect of Replacement of Methionine by Homocystine on Survival of Malignant and Normal Adult Mammalian Cells in Culture

(cancer/metabolic defect/vitamin B12)

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Communicated by I. C. Gunsalus, January 2, 1974

ABSTRACT **In tissue cultures of normal adult and malignant mammalian cells, homocystine has been substituted for methionine in a medium rich in folic acid and cyanocobalamin. Normal adult cells thrive. Three highly malignant cell types from three different species, including man, die.**

A metabolic defect seen to date only in cancer cells was uncovered during an investigation of transfer RNA methylation using tissue cultures of normal adult and malignant cells (1, 2). In several lines of malignant cells we have observed an inability of the cells to survive and grow when methionine is replaced in the medium with homocystine. However, normal adult cell lines derived from mice, rats, and humans survive and grow well with this substitution. The apparent absolute dependence of the malignant cells on preformed methionine is of considerable theoretical interest but may also be of practical importance because it may offer another avenue for treating neoplastic disease.

An earlier study (3), confirmed in our laboratory, has clearly indicated that dietary depletion of methionine has an inhibitory effect on the growth of Walker-256 carcinosarcoma. We became interested in this problem through our studies on methylation of transfer RNA in Walker-256 cells, and the present work demonstrates the dependence of this and other malignant cell lines on preformed methionine. In contrast to the malignant cell lines, we show that a number of normal adult cell lines can satisfy their methionine requirements with homocystine in a folic acid-, cyanocobalamin-rich medium.

METHODS

Tissue cultures of Walker-256 (W-256), breast carcinosarcoma of rats; L1210, lymphatic leukemia of mice; J111, monocytic leukemia of humans; as well as liver epithelial and liver fibroblasts of rats, skin fibroblasts of mice, and fibroblasts from human breast and prostate (all concurrently PPLO negative) were grown in a commercially available, specifically modified McCoy's medium. The composition of the medium was routinely checked by amino-acid analysis before use. It was devoid of bactopeptone but reinforced with 15% fetal-calf serum that had been dialyzed for 24 hr at 4° against three changes of a solution containing 8 g of NaCl, 0.4 g of KCl, 1 g of glucose, and 0.35 g of $NHCO_3$ per liter. In addition, the medium contained trace metals (10 ml/liter of medium of a solution containing $FeSO_4$, 1 mg; $CuCl_2$, 100 μg; $ZnSO_4$, 100 μg; $MnCl_2$, 100 μg/liter) vitamin B12, 2–4 mg/liter; folic acid, 5–10 mg/liter; and either gentamycin (50 μg/ml) or streptomycin sulfate (100 μg/ml) and penicillin G 100 U/ml. Except for subtractions or additions of methionine and homocystine, there were no other alterations in the medium. Two growth curves of each cell type were run simultaneously. One medium contained L-methionine in the usual concentration (15 mg/liter), while the other medium was devoid of methionine, but instead contained either L-homocystine (15 mg/liter) or DL-homocystine (30 mg/liter). Various amounts of vitamin B12, folic acid, homocystine, homocysteine, or the methyl donors—choline, serine, or histidine—were tested using only W-256 cells, but none of these changes altered the basic metabolic defect. Either or both cell counts and total protein determinations (4) were utilized for growth-rate measurements. Cell cultures were usually continued for 5–9 days but in some cases, cells were maintained for 20 days in frequently replenished medium lacking methionine but containing homocystine. At various intervals these cells were placed in unmodified McCoy's medium to check their viability and growth.

To determine the minimal requirement of W-256 cells for methionine, cells were grown in our specifically modified McCoy's medium which contained increasing concentrations of methionine. In addition, each cell type was grown in medium containing methionine (0.1 mM) and added homocystine (0.1 mM) in order to evaluate the possible effect of homocystine on the utilization of methionine.

RESULTS

Striking impairment of the growth of malignant cells is clearly demonstrated when homocystine is substituted for methionine in the growth medium. This is seen in the growth curves of W-256 cells (Fig. 1). A similar result is demonstrated in the experiments on mouse leukemia cells (L1210) (Fig. 2), and on human monocytic leukemia cells (J111) (Fig. 3). Substitution of homocystine for methionine produces little alteration in the growth rates of normal adult epithelial and fibroblastic cells. This is clearly evident in the growth curves of rat-liver fibroblasts (Fig. 4) and of human breast and prostate fibroblasts (Figs. 5 and 6). In each case it is apparent that replacement of methionine by homocystine produces very little or no retardation in the growth rate of normal adult cells, particularly the human lines, whereas in the malignant cells tested growth is unequivocally impaired.

After prolonged incubation (20 days) in media devoid of methionine but with added homocystine, none of the W-256 or L1210 cells remained viable. This was demonstrated by their failure to resume growth when the homocystine medium was replaced with complete McCoy's medium.

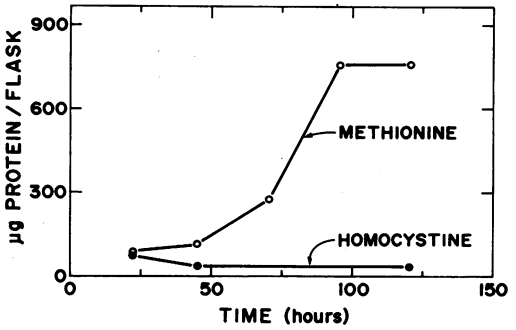

FIG. 1. Growth of W-256 (rat breast carcinosarcoma) cells in culture. Walker-256 cells were grown in two modified McCoy's media, each containing folic acid (10 mg/liter), vitamin B12 (4 mg/liter), and 15% dialyzed fetal-calf serum. One medium contained L-methionine (15 mg/liter); the other medium contained DL-homocystine (30 mg/liter) and no methionine.

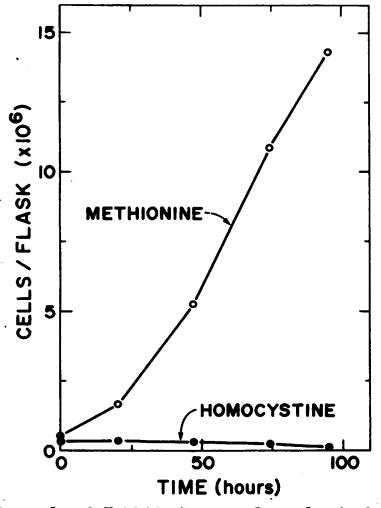

FIG. 2. Growth of L1210 (mouse lymphatic leukemia) cells in culture. The conditions for growth are described in the legend in Fig. 1.

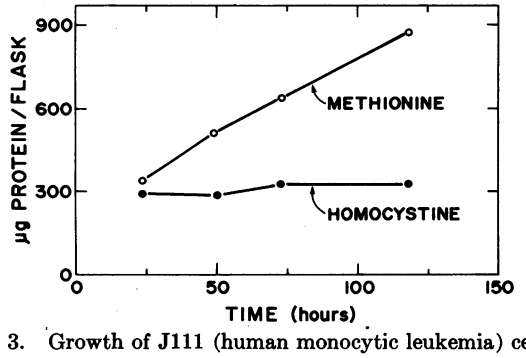

FIG. 3. Growth of J111 (human monocytic leukemia) cells in culture. See legend of Fig. 1 for growth conditions.

In additional experiments we varied the concentration of methionine in the medium in order to assess the absolute dependence for growth on this amino acid. As the concentration of methionine was reduced stepwise, a marked reduction in the growth rate of the W-256 cells was observed (Fig. 7). While

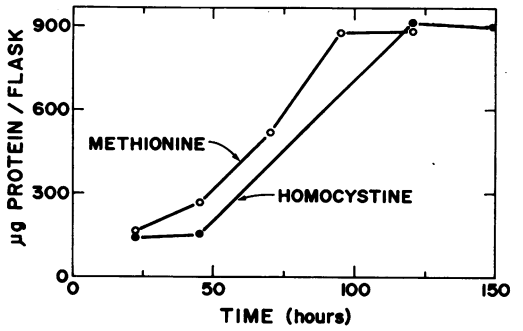

FIG. 4. Growth of rat-liver fibroblasts (L3-16). The conditions for growth were described in the legend of Fig. 1.

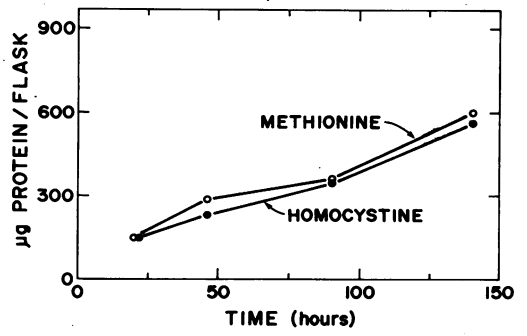

FIG. 5. Growth of human-breast fibroblasts. See the legend of Fig. 1 for growth conditions used.

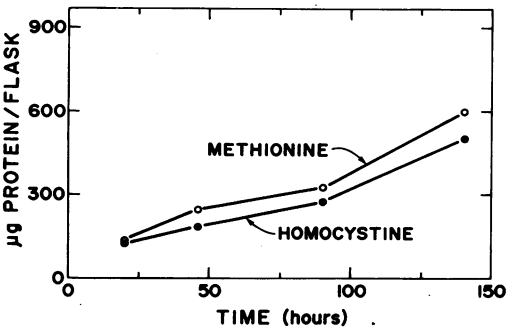

FIG. 6. Growth of human-prostate fibroblasts. The conditions for growth were the same as described in the legend of Fig. 1.

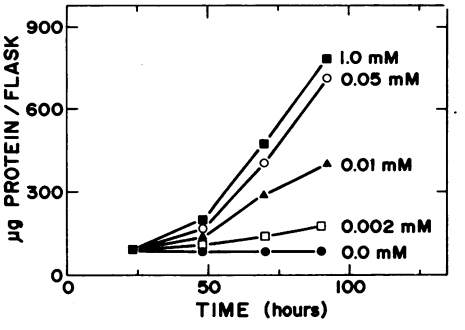

FIG. 7. The dependence of W-256 cell growth in culture on the concentration of methionine in the medium. W-256 cells were grown in modified McCoy's medium containing folic acid (10 mg/liter), vitamin B12 (2 mg/liter), and 15% dialyzed fetal-calf serum. The methionine concentration in the series of media was varied from zero to 1.0 mM.

no growth could be detected in the total absence of methionine, as little as 0.002 mM methionine (which is 2% of the usual concentration found in McCoy's medium) supported growth at 13% of the normal rate. The addition of homocystine (0.1 mM) to the medium containing methionine (0.1 mM) had no effect on the growth rate or morphology of the W-256 cells. As shown in Fig. 7, the W-256 cells were unable to grow in medium totally lacking methionine but containing 15% dialyzed fetal-calf serum; this was true of every cell line tested.

DISCUSSION

Homocystine can replace methionine as an essential nutrient in the medium of mammalian cells growing in tissue culture only when the medium contains both folic acid and vitamin B12 (5, 6). In order for this replacement to be possible, the cells must have the capacity to methylate homocysteine to form methionine. The only enzyme found in mammalian tissue culture cells catalyzing this reaction is 5-methyltetrahydrofolate:homocysteine methyltransferase (7, 8). The catalytic activity of this enzyme has been shown both *in vitro* and *in vivo* to be dependent upon its cofactor vitamin B12 (6, 7, 9). The maximal effect *in vivo* was achieved by a vitamin B12 (hydroxycobalamin) concentration of 0.5 mg/liter (6); however, mammalian cells grow well in media lacking vitamin B12 but not without methionine (10).

Reducing the concentration of methionine in the presence of homocysteine in the tissue cultures of baby hamster kidney (BHK) cells induces increased synthesis of the tetrahydrofolate methyltransferase (8). Maximal growth rate of BHK cells in methionine-deficient medium containing homocysteine and vitamin B12 is achieved by increasing the folic-acid concentration to 0.1 mM (44 mg/liter) (8).

Two tissue culture cell lines derived from human cancers, HEp-2 and HeLa, apparently thrived on a medium without added methionine and choline but containing homocysteine (0.1 mM), 10% undialyzed fetal-calf serum, folic acid (1 mg/liter), and hydroxycobalamin (0.5 mg/liter) (6). However, in tissue culture media lacking methionine but containing vitamin B12 and folic acid, the addition of homocysteine proved inadequate for the growth of L 5178 Y mouse leukemia cells (11).

The dietary requirement of the whole animal (chickens and rats) for methionine can be achieved by feeding homocystine plus either choline or vitamin supplements—specifically vitamin B12 and folic acid (12–15) The formation of methionine occurs in the tissues of the rat and not in the gut, but requires both folic acid and vitamin B12 (16). Moreover the enzyme, 5-methyltetrahydrofolate:homocysteine methyltransferase has been found in all normal adult rat tissues except the small intestine (17). It has also been found in biopsies of normal liver and kidney and cultures of human skin fibroblasts (7, 18, 19).

We have shown, as have others (5, 6, 8, 9), that normal adult mammalian cells can utilize homocystine in place of methionine in tissue culture when the medium contains folic acid (5–10 mg/liter) and vitamin B12 (2–4 mg/liter). We also demonstrated that a rat breast carcinosarcoma (W-256), a mouse leukemia (L1210), and a human monocytic leukemia cannot utilize homocystine for growth even when supplemented with high levels of folic acid and vitamin B12.

The possibility of utilizing this difference between normal and malignant cells is suggested by observing tissue cultures in which fibroblasts and W-256 cells are mixed in the same flask. If the medium contains methionine, the malignant cells invariably outgrow and crowd out the normal fibroblasts. In three weeks this flask contains only thriving cancer cells. On the other hand, if the medium contains homocystine and no methionine, the malignant cells die. The fibroblasts not only live but increase in number, and within 1 week only normal fibroblasts exist within the flask.

In the medium containing cyanocobalamin (2 mg/liter) and folic acid (10 mg/liter) W-256 cells continue to grow when the methionine concentration is reduced from the usual 0.1 mM to 0.01 mM. In order to obtain a cessation of growth, one must reduce the methionine concentration to below 0.002 mM (Fig. 7). To achieve this concentration of methionine in the tissue culture medium, the added fetal-calf serum must be dialyzed.

It is unlikely that all cancers have as complete a metabolic defect as those reported here, but we have used conditions that produce optimal synthesis and activity of the homocysteine methylating enzyme. The concentration of vitamin B12 in our media was 2000–4000 μg/liter, whereas the concentration in normal human serum is 0.2–0.9 μg/liter (20). Similarly, the concentration of folic acid in the medium was 5000–10,000 μg/liter, whereas the concentration in human serum is 6–21 μg/liter (20). It is possible that many tumors may be more susceptible to methionine deprivation at serum levels of vitamin B12 and folic acid.

Throughout all experiments, all neoplastic cell lines remained highly malignant. This was demonstrated by frequent injections of tissue culture cells (10^5 W-256 and L1210) into the animals of origin. The human monocytic leukemia was also malignant.

In order to treat an animal with a malignant tumor, the supply of methionine to the cancer cell must be drastically curtailed. If one applies the results obtained from tissue cultures of W-256 cells, the serum concentration of methionine (normally 0.1 mM) must be reduced more than 50-fold. As previously shown, effective control of tumor growth could not be accomplished by a diet devoid of methionine (3) and, in our own case, by a diet devoid of cystine as well. The eradication of neoplastic cells by methionine deprivation requires a means of destroying or removing the continuously replenished methionine in the serum. This can be accomplished by the enzymatic destruction of the methionine in the serum. Methioninase, an enzyme found in a *Pseudomonas* species (21) and in *Clostridium sporogenes* (22) has already been shown to dethiomethylate and deaminate methionine *in vivo* (23).

The pivotal position of methionine in mammalian metabolism of protein, folic acid, and vitamin B12 (24, 25), particularly when any of the latter three are in insufficient supply, has directed some of our present investigations to the use of methioninase in tissue cultures of normal cells, and in healthy animals that are supplied with nutrients lacking methionine and cystine but fortified with homocystine, vitamin B12, and folic acid. Because it is theoretically possible to nourish every normal cell by supplying homocystine while depleting the neoplastic cells of methionine, we are also treating tumor-bearing animals to determine whether this particular biochemical difference between normal and malignant cells can be exploited therapeutically.

We thank Drs. Robert M. McAllister and Majorie Nicholson of Children's Hospital, University of Southern California, Los Angeles, Calif. 90007 for furnishing us with J111 cells (from human monocytic leukemia peripheral blood) with which they

produced tumors in newborn ATS-treated hamsters. They also verified the persistence of human chromosome counts of this cell line after its passage through the hamster. This work was supported by grants from the Julius and Dorothy Fried Research Foundation and The United States Public Health Service (CA 13196).

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